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Simplified Preparation of Rabbit Fab Fragments

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Papain attached to solid-phase CH-Sepharose 4B was used to digest rabbit IgG. Protein A–Sepharose CL-4B was used to remove undigested IgG and Fc fragments. Pure Fab fragments free of IgG, Fc fragments and papain were readily obtained by this procedure with a yield of about 75%. Polyacrylamide gel electrophoresis of the Fab in the presence of sodium dodecyl sulphate gave a single band under both reducing and non-reducing conditions. The molecular weight of the Fab determined by sedimentation equilibrium was 49,200. Unlike the IgG, the Fab obtained did not form precipitin lines when used in immunoelectrophoresis.

Key words: *rabbit Fab — solid-phase papain — protein A*

Introduction

Fab fragments of IgG have been used in enzyme immunoassay (EIA) instead of IgG (Kato et al., 1976). EIAs of higher sensitivity have been claimed when Fab enzyme is used instead of IgG enzyme. The original method of Porter (1959) is a rather tedious procedure for preparing rabbit Fab fragments, although it is still widely used. Our requirement for such fragments arose from an investigation of the binding site(s) of snake neurotoxin at the neuromuscular junction. For this purpose a Fab enzyme conjugate was needed. We have used solid-phase techniques to simplify the preparation of rabbit Fab fragments.

Materials and Methods

Rabbit IgG

Immune and normal rabbit IgG were prepared by affinity chromatography of crude serum on protien A–Sepharose CL-4B (Pharmacia). The method used was that of Goding (1976) as modified by Coulter et al. (1980). The immune serum had been raised against a neurotoxin (textilotoxin) isolated from the venom of the Australian brown snake, *Pseudonaja textilis*.

Solid-phase papain

Five milligrams of papain (E.C. no. 3.4.22.2) type III, twice recrystallised, obtained from Sigma were reacted with 0.5 g of activated CH-Sepharose 4B (Pharmacia) according to the manufacturer's instructions.

Ninety-one per cent of the papain was covalently bound to the solid phase. Unbound papain was estimated by the Folin–Lowry procedure (Lowry et al., 1951).

The solid phase was stored as a 10% v/v suspension at 4°C in 0.05 M phosphate-buffered saline, pH 7.4 (PBS) containing 0.1% sodium azide.

Preparation of Fab

The procedure used was that described by Hudson and Hay (1976) except for the use of solid-phase papain and protein A to obtain Fab fragments.

The following reagents were added to 10 mg of rabbit IgG in 1 ml of PBS: 0.2 ml of 16 mg/ml cysteine hydrochloride, 0.2 ml of 8 mg/ml EDTA, sodium salt, 1 ml of solid-phase papain (equivalent to 1 mg), washed with 0.15 M phosphate, pH 7.0, to remove sodium azide.

The mixture was incubated with gentle stirring at 37°C for 4 h. The solid phase was sedimented by gentle centrifugation ($1200 \times g$ for 5 min) and the supernatant washed through a 4 ml protein A–Sepharose CL-4B column with 10 ml of PBS (pool A). Undigested IgG and Fc fragments were eluted from the column by successive washings with: 10 ml of PBS containing 0.1% Tween 20: this was discarded. Ten millilitres of PBS, discarded. Ten millilitres of 0.1 M glycine/HCl containing 1 M NaCl, pH 3. This was collected into 10 ml of PBS and adjusted to pH 7.4 with 1 M NaOH (pool B).

The column was finally stored in PBS containing 0.1% sodium azide.

Pools A and B were concentrated separately to about 5 ml by ultrafiltration over a PM-10 membrane in a model 52 cell (Amicon).

IgG and Fab concentrations were determined with values of $E^{1\%}_{279\,nm} = 14.0$ and 14.8 respectively (Mandy and Nisonoff, 1963).

Immunoelectrophoresis (IEP)

IEP was performed by the method of Scheidegger (1955). The antiserum used was rabbit anti-textilotoxin.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed as described by Weber and Osborn (1969) and Laemmli (1970). SDS was obtained from British Drug Houses as were acrylamide and methylenebisacrylamide. TEMED and 2-mercaptoethanol (2-ME) were from Eastman Organic Chem. Protein molecular weight standards were obtained from Pharmacia.

Analytical ultracentrifugation

The molecular weight determination was done by meniscus depletion in a Beckman model E analytical ultracentrifuge. The method of Yphantis (1964) was used. A $\bar{v}$ value of 0.73 cc/g was assumed for rabbit Fab.

Enzyme immunoassay (EIA)

EIA was used to determine the specific antibody titre after preparation of Fab from IgG. The EIA was as described previously (Coulter et al., 1981) for direct assay of snake venom. The wells of polystyrene microhaemagglutination plates (u-wells, Cooke Lab. Prod.) were coated with textilotoxin. The following procedure was used.

Plates were floated in a water bath at 37°C for 2 h after adding 0.1 ml of 1 μg/ml textilotoxin in 0.05 M sodium carbonate buffer, pH 9.6, to the wells. The plates were washed and incubations performed as previously described (Coulter et al., 1981). The relative ability of rabbit IgG anti-textilotoxin and Fab anti-textilotoxin to block the uptake of rabbit IgG anti-textilotoxin–horse radish peroxidase (HRPO) conjugate by the polystyrene bound textilotoxin was determined in the following way. Serial dilutions of IgG and Fab were incubated in wells coated with textilotoxin for 30 min at 37°C. After washing and subsequent reaction with conjugate the degree to which IgG and Fab had blocked conjugate uptake was determined by intensity of colour development on addition of substrate to the wells.

Mouse protection assays

IgG and Fab anti-textilotoxin were tested for their ability to neutralise the lethal effects of textilotoxin in mice. Eighteen to 21 g Swiss mice (CSL strain) were used in the assay. Four mice were used at each dose level and all injections were given intravenously. Four LD_{50} of textilotoxin were incubated with serial dilutions of immune IgG and Fab. All doses were given in a volume of 0.2 ml. A 0.1% solution of bovine serum albumin in 0.85% NaCl was used as diluent; this reduces non-specific adsorption of textilotoxin (Broad et al., 1979).

IgG and Fab prepared from normal rabbit IgG were used as controls.

Ion exchange chromatography of Fab prepared by the solid-phase papain/protein A procedure

Fab prepared by the solid-phase papain/protein A procedure was subjected to ion exchange chromatography. The method of Porter (1959) as modified by Hudson and Hay (1976) was used.

Results and Discussion

Fab fragments prepared by the solid-phase papain/protein A procedure gave a single diffuse band on SDS-PAGE run under non-reducing conditions. A single band of M_r 27,000 was obtained on SDS-PAGE run under reducing conditions (Fig. 1). Rabbit Fab and Fc fragments have M_rs of about 50,000 (Marler et al., 1964). No IgG was detectable by SDS-PAGE in the Fab preparation.

A M_r of 49,200 was obtained by sedimentation equilibrium. The M_r was constant through most of the solution column, with a slight increase towards the base of the cell.

Dialysis of the Fab preparation against a nominal 10,000 M_r cut-off membrane ensures removal of low M_r products. Apart from the Fab band, no other high or low

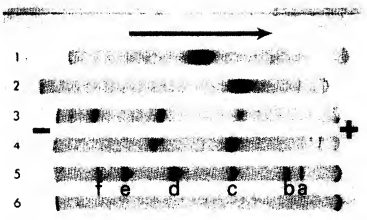

Fig. 1. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (Laemmli, 1970) of rabbit IgG and its fragments resulting from solid-phase papain digestion. Essentially the same results were obtained with the Weber and Osborn (1969) gel system (not shown). Gel 1: Non-protein A bound material after solid-phase papain digestion, i.e., Fab run under non-reducing conditions. Gel 2: Fab prepared by the solid-phase papain procedure. Sample reacted with 2-ME. Gel 3: protein A bound components after solid-phase papain digestion. These were eluted at low pH. Gel run under non-reducing conditions. Gel 4: sample as for gel 3, treated with 2-ME. Gel 5: protein M_r standards: a, α-lactalbumin, 14,400; b, trypsin inhibitor, 20,100; c, carbonic anhydrase, 30,000; d, ovalbumin, 43,000; e, albumin, 67,000; f, phosphorylase b, 94,000. Gel 6: blank gel.

M_r products were visible by SDS-PAGE.

When the Fab preparation was fractionated by ion exchange, 94% of the starting material was recovered. Sixty-five per cent of the starting material was eluted with the equilibrating buffer (0.01 M sodium acetate, pH 5.5) and 29% was eluted before a sodium acetate concentration of 0.2 M was reached. No other material was eluted, even when 1 M sodium acetate, pH 5.5, was used.

When the anti-textilotoxin titre of the Fab preparation was compared with that of IgG by EIA, the Fab concentration which yielded 50% of full colour in the assay was 4.5 μg/ml. The IgG concentration which gave the same colour was 6.2 μg/ml. In terms of anti-textilotoxin activity in the EIA, when their relative M_rs are considered, the Fab and IgG were practically equivalent. The final yield of Fab was always about 75% of that theoretically possible, i.e., 5 mg Fab from 10 mg of IgG.

The neutralisation tests performed in mice showed that on a weight basis the IgG and Fab anti-textilotoxin preparations were equivalent in neutralising ability, i.e., a loss of approximately 30% of specific antibody activity.

When equivalent concentrations of Fab and IgG anti-textilotoxin were tested by IEP against textilotoxin, precipitin lines were obtained with IgG but not with the Fab preparation.

These results demonstrate that Fab fragments can be obtained from rabbit IgG with losses of 20–30% of initial IgG antibody activity. The steps involved in the procedure are relatively simple. The solid-phase papain is not difficult to prepare;

the reaction with IgG is straightforward as in the protein A purification of the Fab. The procedure represents a marked improvement in terms of simplicity and time saving over the ion exchange procedure for the preparation of rabbit Fab fragments.

Note Added in Proof

Since completing this paper an important article came to our attention describing the same experimental procedure:

Goding, J.W., 1978, J. Immunol. Methods 20, 241.

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